

**Small RNAs and Small Proteins Involved in Resistance to Cell Envelope Stress and Acid
Shock in *Escherichia coli*: Analysis of a Bar-coded Mutant Collection**

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1 More than 80 small, regulatory RNAs (sRNAs) and 60 proteins of 16-50 amino acids
2 (small proteins) are encoded in the *E. coli* genome. The vast majority of the corresponding
3 genes have no known function. We screened 125 DNA bar-coded mutants to identify novel
4 cell envelope stress and acute acid shock phenotypes associated with deletions of genes
5 coding for sRNAs and small proteins. Nine deletion mutants (*ssrA*, *micA*, *ybaM*, *ryeF*,
6 *yqcG*, *sroH*, *ybhT*, *yobF*, and *glmY*) are sensitive to cell envelope stress and two are resistant
7 (*rybB* and *blr*). Deletion mutants of genes coding for four small proteins (*yqgB*, *mgrB*,
8 *yobF*, and *yceO*) are sensitive to acute acid stress. We confirmed each of these phenotypes
9 in one-on-one competition assays against otherwise wild-type *lacZ* mutant cells. A more
10 detailed investigation of the SsrA phenotype suggests that ribosome release is critical for
11 resistance to cell envelope stress. The bar-coded deletion collection we generated can be
12 screened for sensitivity or resistance to virtually any stress condition.

1 Small, regulatory RNAs (sRNAs) play critical regulatory roles in all domains of life.
2 Numerous approaches have been taken to discover sRNA-encoding genes in bacteria (reviewed
3 in references (1, 27)), including bioinformatic searches for conservation as well as promoter and
4 Rho-independent terminator sequences in intergenic regions. sRNAs have also been detected
5 directly by sequencing or microarray analysis, often after size selection or co-
6 immunoprecipitation with RNA-binding proteins. Approximately 80 sRNAs have been
7 identified in *E. coli*. A few sRNAs bind proteins to effect a cellular response, but the vast
8 majority of sRNAs characterized to date act by base pairing with mRNAs (reviewed in reference
9 (53)). sRNA base pairing with an mRNA can bring about any of a number of outcomes:
10 exposing or occluding a ribosome binding site; increasing or decreasing mRNA stability; or
11 terminating transcription.

12 Those sRNAs whose functions have been delineated regulate a wide array of
13 physiological responses (reviewed in reference (53)). For example in *E. coli*, sRNAs are induced
14 to promote translation of a stationary phase-specific σ factor, to downregulate σ^{70} -RNA
15 polymerase activity at certain promoters in stationary phase, and to induce and repress genes in
16 response to iron availability (53). In *Vibrio* species, sRNAs act to integrate quorum sensing
17 signals (53). Many Gram-negative bacteria also employ sRNAs to regulate the composition of
18 outer membrane proteins (OMPs) within their cell envelopes (reviewed in references (17, 51)).

19 In work growing out of our screens for sRNAs, we have also initiated searches for
20 unannotated genes encoding proteins between 16 and 50 amino acids in length (19).
21 Approximately 60 genes have been shown to encode small proteins in *E. coli* (19). Very little is
22 known about what the vast majority of small proteins do. However, the few whose functions
23 have been elucidated act in a number of roles: as intercellular signals to regulate the onset of

1 genetic competence in Gram-positive bacteria (7); as intracellular toxins (12) and antibiotics (23)
2 in various bacteria; and as kinase inhibitors in *Bacillus subtilis* (40).

3 sRNAs and small proteins of known function play diverse cellular roles, so how can
4 those of unknown function be analyzed most efficiently? One approach is to uncover
5 phenotypes associated with deletions of sRNA- and small protein-coding genes. The existence
6 of a deletion phenotype indicates that a sRNA or small protein performs a biologically relevant
7 function that is amenable to study in the laboratory. Aside from demonstrating the physiological
8 relevance of the gene, the discovery of a deletion phenotype greatly facilitates the study of the
9 corresponding sRNA or small protein by further genetic analysis. Biochemical and cytological
10 approaches also are aided by knowledge of whether tagged or mutant derivatives complement a
11 mutant phenotype.

12 Thus far, very little has been done to systematically associate deletion phenotypes with
13 genes coding for bacterial sRNAs or small proteins. However, a number of studies have been
14 undertaken to identify phenotypes tied to the absence of other genes in *E. coli*. Many of these
15 investigations have made use of the Keio collection, a set of approximately 3,900 deletions of
16 nonessential genes in *E. coli*, which contains relatively few deletions of sRNA- and small
17 protein-coding genes (2). This collection has been screened for mutants deficient in biofilm
18 formation (32) and in resistance to various antibiotics (45). Two groups have also exploited
19 bacterial conjugation to identify synthetically lethal interactions in a high-throughput manner (5,
20 48). Others have employed customized microarrays to analyze the Keio collection in batch
21 competition experiments (43). In this approach (known as Monitoring of Gene Knockouts or
22 MGK), every strain in the collection is mixed and subjected to mock and stress treatments.

1 Individual strains are subsequently enumerated by quantifying DNAs amplified from the regions
2 flanking every antibiotic resistance cassette on a custom microarray.

3 The yeast community has created a knockout collection of approximately 5,900 yeast
4 genes (13). However, unlike the Keio collection, every strain in the yeast deletion collection
5 contains two unique 20-mer DNA bar codes (13, 35). These bar codes enable the execution of
6 parallel screens for deletion phenotypes in large-scale competition experiments using
7 standardized microarrays. We have co-opted this methodology to create a series of 125 DNA
8 bar-coded deletion mutants in *E. coli* (Fig. 1). We employed this collection to identify deletion
9 mutants of genes coding for sRNAs and small proteins that are sensitive or resistant to cell
10 envelope stress or to acute acid stress, two conditions *E. coli* encounters during its life cycle as a
11 pathogen or symbiont in higher eukaryotes (i.e. acid stress in the stomach and cell envelope
12 stress in the intestine).

MATERIALS AND METHODS

Media and media supplements. Luria-Bertani broth (10 g of tryptone, 5 g of yeast extract, 10 g of NaCl per liter) was prepared from a pre-mixed stock (Invitrogen, Lot #A08-23). M63 minimal medium [15.2 mM (NH₄)₂SO₄, 22.1 mM KH₂PO₄, 40.3 mM K₂HPO₄, 1 mM MgSO₄, 3.30 μM FeSO₄] was supplemented with 5% sucrose (w/v), 0.2% glycerol (w/v), 5 mg/l vitamin B1, and 1 mg/l biotin. When necessary, antibiotics were used at the following concentrations: kanamycin, 30 μg/ml; chloramphenicol, 25 μg/ml; ampicillin, 100 μg/ml; carbenicillin, 100 μg/ml; tetracycline, 12.5 μg/ml. Isopropyl β-D-1-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) were used at a final concentrations of 1 mM and 100 μg/ml, respectively.

Strains and oligonucleotides. All strains are derivatives of the laboratory stock of *E. coli* K-12 MG1655. The strains and oligonucleotides used in the study are listed in Tables S1 and S2, respectively. Platinum Taq DNA Polymerase High Fidelity (Invitrogen) was employed in all PCR reactions.

Generation of bar-coded kanamycin resistance cassettes. Bar-coded kanamycin resistance cassettes were generated by a two-step PCR process. First, P1 and P2 primers were used to amplify the kanamycin resistance cassette from pKD13 (8). The P1 and P2 primers contained common priming sequences, unique 20-mer “UP” and “DN” DNA barcodes specific to each locus being deleted, and regions complementary to the kanamycin resistance cassette. P3 and P4 primers containing DNA sequences homologous to the regions flanking the locus to be deleted as well as DNA sequences complementary to the 5'-ends of the first PCR product were used to amplify the gel-purified first round reaction products in a second round PCR. The reaction products from the second round PCR were incorporated into the chromosome by mini-

1 λ -Red-mediated recombination (8, 57). The bar-coded kanamycin cassettes were moved to a
2 fresh genetic background (wild-type *E. coli* K-12 MG1655 cells) by P1 transduction (46) and
3 sequenced. For further characterization of the mutant strains, the kanamycin antibiotic resistance
4 cassettes were excised from the chromosome by Flp-mediated recombination (6). In general,
5 genes encoding sRNAs with mapped 5'- and 3'- ends and open reading frames (inclusive of the
6 stop codon) encoding small proteins were deleted in their entirety. When possible, care was
7 taken to avoid deleting flanking genes or their regulatory elements; however, intergenic regions
8 containing sRNAs with unmapped 5'- and 3'-ends were deleted completely.

9 **Generation of complementation constructs.** The counter-selectable *cat-sac* cassette
10 (25) was PCR-amplified using primers ECH938 and ECH495, which also carried sequences
11 homologous to regions upstream (123 to 177 base pairs before the start codon) and downstream
12 (1 to 55 base pairs after the stop codon) of *lacZ* on the *E. coli* chromosome. The purified PCR
13 product was used in conjunction with the mini- λ -Red system (8, 57) to replace *lacZ* with the *cat-*
14 *sac* cassette and create GSO291.

15 Complementation constructs were generated by PCR-amplifying the appropriate loci with
16 primers that contained the *lacZ*-flanking regions described above. *ssrA* alleles were similarly
17 integrated at *lacZ* after being amplified from pJW28 (*ssrA*⁺) (38) and pJW29 (*ssrA*⁰) (38) using
18 primers ECH1218 and ECH1227. The mini- λ -Red system was employed to replace the *cat-sac*
19 cassette with each complementation construct. A control *lacZ* deletion strain was created by
20 using the primers ECH1012 and ECH1013 to PCR-amplify two complementary oligonucleotides
21 (ECH1007 and its complement) in which the upstream and downstream *lacZ* flanking regions
22 had been fused together. The resulting PCR product was used to replace the *cat-sac* cassette.
23 Transformants were grown on M63 minimal medium supplemented with 5% sucrose to select for

1 cells that lost the *cat-sac* cassette. All complementation constructs were confirmed by
2 sequencing.

3 **Screening bar-coded deletion collection for novel phenotypes.**

4 **(i) Cell growth.** Cells from each bar-coded deletion strain were inoculated separately
5 into 50-ml conical tubes containing 5 ml of LB broth and grown for 16 h at 37°C with shaking
6 (250 rpm).

7 For the cell envelope stress screen, the overnight cultures ($OD_{600} \sim 5.5$) were pooled and
8 used to inoculate 30 ml of pre-warmed (37°C) LB broth at a dilution of 1:2000. The culture was
9 split into two 15-ml subcultures. Cell envelope stress was imposed in one subculture by adding
10 sodium dodecyl sulfate (SDS) (final concentration 0.025% [w/v]) and EDTA (pH 8.0) (final
11 concentration 1 mM). Both 15-ml subcultures were incubated in a shaking (250 rpm) water bath
12 at 37°C. A 1-ml aliquot of cells was harvested from the mock-treated subculture when the OD_{600}
13 was between 0.280 and 0.400. A 1-ml aliquot of cells was collected from the cell envelope stress
14 culture when the OD_{600} was between 0.280 and 0.400 and within 0.05 OD units of the OD_{600}
15 achieved by the mock-treated cells at the time of their harvesting.

16 For the acid shock screen, the overnight cultures were pooled and the OD_{600} of this
17 pooled culture was determined ($OD_{600} \sim 5.5$). Two 1-ml aliquots of the mixed culture were
18 placed into 1.5-ml Eppendorf tubes. One aliquot was acidified to pH 1.8 with an aqueous
19 solution of 37% (w/v) HCl. Both the mock-treated and acid-treated cells were incubated in a
20 tabletop heating block at 37°C with shaking (1400 rpm) for 10 min. Cells were subsequently
21 washed 3 times with 1 ml of 1x phosphate-buffered saline (PBS) (pH 7.4). The mock-treated
22 and acid-treated cells were inoculated (1:5000) into separate 250-ml flasks each containing 30 ml
23 of pre-warmed (37°C) LB broth. Both cultures were incubated in a water bath (37°C, 250 rpm)

1 until they achieved an OD₆₀₀ within 0.2 OD units of the original mixed culture, at which point in
2 time a 1-ml aliquot of cells was harvested.

3 **(ii) Hybridization and scanning of microarray.** UP and DN bar codes from each
4 sample were quantified on a Genflex Tag 16K Array v2 (Affymetrix) (36). The methods
5 summarized here are described in greater detail by Pierce and colleagues (36). Recipes for
6 making 12x MES stock solution, 2x hybridization buffer, hybridization mix, wash A solution,
7 wash B solution, and biotin staining solution as well as a step-by-step protocol for hybridizing
8 DNA bar codes to the microarray can also be found in the Supplementary Materials.

9 First, genomic DNA was prepared from each sample using a Wizard Genomic DNA
10 Purification Kit (Promega). UP bar codes were PCR-amplified using primers ECH361 and
11 ECH427. DN bar codes were PCR-amplified using primers ECH362 and ECH428. ECH427
12 and ECH428 were biotinylated at their 5'-ends. Approximately 0.2 µg of genomic DNA was
13 used as a template in each reaction.

14 Second, each microarray was filled with 140 µl of 1x hybridization buffer and incubated
15 (42°C, 20 rpm) in an Affymetrix GeneArray Hybridization Oven for 10 min. The 1x
16 hybridization buffer was subsequently removed from each microarray and replaced with a
17 solution (previously boiled for 2 min and incubated on ice for 2 min) consisting of 30 µl of the
18 UP and DN bar code PCRs combined with 90 µl of hybridization mix. The arrays were then
19 rotated at 20 rpm in the hybridization oven for 10-16 h at 42°C.

20 The following day, the hybridization mix was removed, and each microarray was washed
21 twice with wash A solution (room temperature), six times with wash B solution (42°C), and once
22 with wash A solution (room temperature). Then the wash A solution was aspirated and replaced
23 with biotin staining mix. Each microarray was rotated at 20 rpm for 10 min at 42°C. The arrays

1 were washed 6 times with wash solution A (room temperature). The arrays were then filled with
2 wash A (room temperature) and scanned at an emission wavelength of 560 nm with an
3 Affymetrix GeneArray Scanner.

4 **(iii) Analysis of array data.** UP bar codes were analyzed separately from DN bar codes.
5 GeneChip Operating Software (Affymetrix) was used to extract the arbitrary fluorescence values
6 associated with each probe. Every bar code is queried by five probes on the microarray. The
7 arbitrary fluorescence intensities associated with the individual probes in these quintets were
8 averaged to yield a mean fluorescence intensity for each bar code. The background fluorescence
9 intensity was determined by averaging the fluorescence intensities of probes associated with bar
10 codes that were not present in any strain. The background fluorescence intensity was subtracted
11 from the mean fluorescence intensity of each bar code. Bar codes with a background-corrected
12 mean intensity of less than 200 arbitrary fluorescence units in the mock-treatment sample were
13 excluded from further analysis. One caveat to this approach is that the signal intensity observed
14 for a bar code on the array does not scale in a linear manner with the actual concentration of the
15 bar code in solution (35, 36). As a consequence, the difference in bar code concentrations
16 between two samples tends to be underestimated in the final array analysis. As previously
17 described, the remaining mean bar code fluorescence intensities were multiplied by a correction
18 factor ($e^{0.00031 \times \text{mean bar code intensity}}$) to account for this effect (35, 36). The resulting corrected
19 fluorescence intensity associated with each bar code in stress-treated cells was divided by its
20 fluorescence intensity in mock-treated cells to obtain a relative abundance (R.A.) value. Both
21 experiments were performed in triplicate, giving rise to three unique sets of UP and DN bar code
22 R.A. values for each stress condition.

1 **One-on-one competition assays.** Cells from strains to be tested were inoculated
2 separately into 50-ml conical tubes containing 5 ml of LB broth and grown for 16 h at 37°C with
3 shaking (250 rpm). An aliquot of the overnight cultures of each deletion mutant was mixed with
4 an equal amount of the overnight culture of the *ΔlacZ* (NM601) cells.

5 For the cell envelope stress assays, each of the mixed cultures was used to inoculate 30
6 ml of pre-warmed (37°C) LB broth at a dilution of 1:2000. The 30-ml culture was split into two
7 15-ml subcultures. Cell envelope stress was imposed in one subculture by adding SDS (final
8 concentration 0.025% [w/v]) and EDTA (pH 8.0) (final concentration 1 mM). Both 15-ml
9 subcultures were incubated in a shaking (250 rpm) water bath at 37°C. For the mock-treated
10 subculture, cells were harvested when the OD₆₀₀ was between 0.280 and 0.400. For the cell
11 envelope stress subculture, cells were collected when the OD₆₀₀ was between 0.280 and 0.400
12 and within 0.05 OD units of the OD₆₀₀ achieved by the mock-treated cells at their time of
13 harvesting.

14 For the acid shock assays, two 1-ml aliquots of the mixed culture were placed into 1.5-ml
15 Eppendorf tubes. One aliquot was acidified to pH 1.8 with an aqueous solution of 37% (w/v)
16 HCl. Both the mock-treated and acid-treated cells were incubated in a tabletop heating block at
17 37°C with shaking (1400 rpm) for 10 min. Cells were subsequently washed 3 times with 1 ml of
18 1x phosphate-buffered saline (PBS) (pH 7.4).

19 Aliquots of mock- and stress-treated subcultures were diluted appropriately and spread on
20 LB agar plates containing IPTG and X-Gal. After overnight incubation at 37°C, the number of
21 blue and white colonies arising from the mock treatment and the stress treatment samples were
22 scored to obtain a competitive index (C.I.).

RESULTS

Generating bar-coded deletion strains. The phenotyping of individual bacterial strains under numerous stress and growth conditions is time- and labor-intensive. The effort involved is compounded when hundreds or thousands of strains need to be screened simultaneously. An alternative methodology is to perform batch competition experiments in which all strains are mixed together and subjected to selective pressure. In this approach, conditions can be manipulated to select for extremely resistant strains. However, it is difficult to identify sensitive or moderately resistant mutants without a means to enumerate the number of cells corresponding to each strain within the population.

We incorporated unique 20-mer DNA sequences (bar codes) into a collection of 125 directed deletion mutants. These bar codes can be used with microarray analysis to allow the quantification of individual strains within large-scale competition experiments. At the time we performed the large-scale competition experiments described below, this collection contained 122 strains. 47 of these strains were single deletion mutants of genes encoding sRNAs and 50 strains were deletion mutants of genes encoding small proteins of 50 amino acids or less. Three additional strains were also created that are deleted for the repetitive *sib* and *ldr* loci ($\Delta s i b A B C D E$, $\Delta l d r A B C$, and $\Delta l d r A B C D$). Thirteen strains are deleted for genes encoding proteins between 50 and 70 amino acids in length, and eight control strains are deleted for genes known to be required for survival under various stress conditions (e.g. *smmA*, *gadE*, *trpA*, *uspA*, *uspB*, *uspD*, *uspE*, and *oxyR*). One final strain is deleted for *dppA*, a target of the GcvB sRNA (50).

Homologous recombination was employed to replace the genes listed above with antibiotic resistance cassettes flanked by two unique bar codes (Fig. 1). Common priming sites

1 were also incorporated upstream and downstream of each bar-coded antibiotic resistance
2 cassette. Hence, all of the upstream (UP) and downstream (DN) bar codes in a population of
3 cells could be amplified in two separate PCR reactions by using the UP and DN common primers
4 in conjunction with primers designed to anneal to the antibiotic resistance cassette. One caveat
5 of this strategy is that any phenotypes we uncovered could arise as a consequence of polarity
6 effects imposed by the antibiotic resistance cassettes on downstream genes. To minimize this
7 potential problem, the antibiotic resistance cassettes were excised from strains that were
8 subjected to further analyses. The bar code and common primer sequences left behind after
9 excision of the antibiotic resistance cassette are designed to limit cross hybridization, and should
10 not give rise to any significant secondary structures that would affect downstream gene
11 expression.

12 **Strains sensitive to cell envelope stress.** Two pieces of information led us to
13 hypothesize that it would be fruitful to screen our deletion collection for cell envelope stress
14 phenotypes generated by exposure to SDS and EDTA. First, several sRNAs regulate the
15 synthesis of outer membrane proteins (OMPs) in bacteria (reviewed in references (17, 51)).
16 Second, it has been reported that up to 70% of the small proteins in *E. coli* are predicted to be
17 membrane-localized (19).

18 The bar-coded deletion collection was subjected to mock treatment and to cell envelope
19 stress as described in the Materials and Methods. UP and DN bar codes were amplified from the
20 genomic DNAs of mock- and stress-treated cells and were hybridized to a microarray containing
21 complementary probes. A relative abundance value (R.A.) was obtained for each bar code by
22 dividing its average stress treatment array intensity by its average mock treatment array intensity.
23 An R.A. = 1 indicates that a deletion mutant has no phenotype. An R.A. < 1 indicates that a

1 mutant is sensitive to the stress being imposed, while an R.A. > 1 indicates that a mutant is
2 resistant to the stress.

3 A representative histogram plot of R.A. values obtained from one cell envelope stress
4 screening experiment is shown in Fig. 2. The vast majority of the deletion mutants had an R.A.
5 close to one, indicating that they are wild-type with respect to cell envelope stress. The cell
6 envelope stress screening experiments were performed in triplicate. Due to the fact that each
7 strain contains two unique bar codes, two independent R.A. measurements can be calculated for
8 every deletion mutant within the population. Thus, six R.A. measurements were obtained for
9 each deletion mutant. Although the R.A. values calculated for any particular deletion mutant
10 differed across the three trials (Table S3), the rank orders for the most sensitive and resistant
11 strains were similar between experiments. A number of deletion mutants appeared repeatedly in
12 the list of the twenty most sensitive strains (i.e. those with the lowest twenty R.A. values) (Table
13 S3); those that were among the twenty most sensitive strains in at least four of six R.A.
14 measurements were analyzed further. As expected (42), the *smpA* deletion mutant was the most
15 sensitive strain in every cell envelope stress experiment. Ten other deletion strains (*ssrA*, *ybaM*,
16 *mica*, *ryeF*, *yqcG*, *yobF*, *sroH* *ybhT*, *yqgB*, and *glmY*) were also sensitive to growth in SDS and
17 EDTA in at least four of the six measurements.

18 **Strains resistant to cell envelope stress.** The R.A. measurements were also analyzed
19 for strains that might be resistant to cell envelope stress. As with the potentially sensitive strains,
20 some deletion mutants appeared repeatedly among the most resistant strains (Table S3).
21 Deletion mutants of genes coding for one sRNA (*rybB*) and one small protein (*blr*) ranked with
22 the five highest R.A. measurements at least four of six times and were analyzed further. The
23 *ybgT* deletion mutant also appeared within this set, but its apparently intrinsic resistance to cell

1 envelope stress is difficult to interpret and may be misleading given that *ybgT* cells grow very
2 poorly on LB agar plates and in LB broth at 37°C (data not shown). As such, we did not analyze
3 this potential phenotype further.

4 Although a combination of SDS and EDTA has been used previously to impose cell
5 envelope stress (42), it should be noted that the phenotypes uncovered in the cell envelope stress
6 screen may not have arisen as a consequence of cell envelope stress *per se*. Another possibility
7 is that the cells are responding to the depletion of available divalent cations from the media.

8 **Verification of cell envelope stress phenotypes.** The phenotypes of putatively sensitive
9 or resistant strains were verified in one-on-one competition assays with otherwise wild-type *lacZ*
10 mutants. In contrast to the large-scale screens, these experiments were conducted with deletion
11 strains where the antibiotic resistance cassettes incorporated at each deletion locus had been
12 excised by Flp-mediated recombination. The one-on-one competition assays were conducted by
13 mixing LacZ⁺ deletion mutant cells of interest (competitor strain) with otherwise wild-type LacZ⁻
14 cells (reference strain) and subjecting one half of this mixture to a mock treatment and the other
15 half to the cell envelope stress conditions described above. Cells from each sample were
16 incubated on LB plates supplemented with IPTG and X-Gal. The numbers of blue and white
17 colonies on these plates were scored. A competitive index (C.I.) was obtained by dividing the
18 ratio of competitor cells to reference cells observed on the stress treatment plates by the ratio of
19 competitor cells to reference cells observed on the mock treatment plates. Sensitive strains
20 exhibit a C.I. less than one, while resistant strains have a C.I. greater than one.

21 The results of four representative competition experiments are displayed in Fig. 3. The
22 mock samples in each experiment contain blue and white cells in roughly equal proportions. The
23 first panel shows that *blr* mutants are more resistant to growth in SDS and EDTA than wild-type

1 cells, as evidenced by the increased ratio of blue to white cells after cell envelope stress
 2 treatment (C.I. > 1). When wild-type MG1655 was employed as a competitor strain, the ratio of
 3 blue to white cells remained unchanged after stress treatment, indicating that a deletion of *lacZ*
 4 does not affect *E. coli* fitness in either a positive or negative manner in this assay (Fig. 3 and Fig.
 5 4). *sroH* and *ssrA* mutant cells exhibit increasingly severe sensitivity phenotypes, which is
 6 reflected in the decreasing ratios of blue to white cells after stress treatment (C.I. < 1).

7 One-on-one competition experiments were performed in triplicate with each of the
 8 putatively resistant or sensitive strains identified in the large-scale cell envelope stress assays
 9 (Fig. 4). After the $\Delta smpA$ control strain (data not shown), $\Delta ssrA$ and $\Delta micA$ cells had the most
 10 severe cell envelope stress phenotypes (C.I. close to zero) (Fig. 4A). *ybaM*, *ryeF*, and *yqcG*
 11 deletion mutants were also very sensitive (C.I. values between 0.1 and 0.2) (Fig. 4A). *sroH*,
 12 *ybhT*, *yobF*, and *glmY* deletion mutants (C.I. values between 0.3 and 0.6) were only moderately
 13 sensitive. *yggB* deletion mutants were not sensitive to cell envelope stress. In total, nine of the
 14 ten putatively sensitive strains exhibited significant phenotypes in one-on-one competition assays
 15 with LacZ⁻ cells. Five of these nine strains were deleted for genes encoding sRNAs (*ssrA*, *micA*,
 16 *ryeF*, *sroH*, and *glmY*), three were deleted for genes encoding small proteins (*yqcG*, *ybhT*, and
 17 *yobF*), and one was deleted for a gene encoding a 53 amino acid protein (*ybaM*). Finally, the
 18 resistance phenotypes exhibited by *rybB* and *blr* deletion mutants were also confirmed (C.I.
 19 values of 2.2 and 3.4, respectively) (Fig. 4B).

20 **Complementation of select cell envelope stress phenotypes.** We examined whether the
 21 deleted gene was responsible for the phenotypes of the three deletion mutants most sensitive to
 22 cell envelope stress (*ssrA*, *ybaM*, and *micA*) as well as that of a more moderately sensitive
 23 deletion mutant (*ybhT*) by performing complementation experiments. To accomplish this, each

1 of these genes was integrated under the control of its own promoter at the *lacZ* locus of the
2 appropriate deletion mutant. The *priC* gene immediately upstream of *ybaM*, was also included in
3 the *ybaM* complementation construct. Each of these strains was subjected to cell envelope stress
4 competition experiments against wild-type MG1655 cells. As shown in Fig. 4C, deletion
5 mutations at *ssrA*, *ybaM*, *micA*, and *ybhT*, could be complemented as evidenced by the fact that
6 all of the complemented strains exhibit C.I. values close to one. We proceeded to further
7 characterize the two strains with the most severe phenotypes, $\Delta ssrA$ and $\Delta micA$.

8 **SsrA function is required for cell envelope stress resistance.** *ssrA* encodes a
9 specialized RNA (tmRNA) that frees stalled ribosomes from mRNA transcripts (reviewed in
10 (10)). During this process, a portion of SsrA that encodes a proteolysis tag is inserted into the
11 ribosome concomitantly with displacement of the mRNA transcript. This tag is translated as the
12 C-terminus of the nascent polypeptide chain, and targets the protein for degradation. Proteolysis
13 of SsrA-tagged proteins is carried out primarily by the ClpXP protease (26). This is
14 demonstrated by the fact that SsrA-tagged proteins accumulate and can be readily detected by
15 immunoblot analyses in $\Delta clpX$ and $\Delta clpP$ cells, but not in deletion mutants of genes coding for
16 other major cellular proteases (26).

17 To test if the freeing of stalled ribosomes from mRNAs and aborted polypeptides is
18 sufficient for resistance or if both ribosome release and proteolysis tagging are required for cell
19 envelope stress resistance, we determined the phenotype of $\Delta clpP$ cells. If the proteolysis of
20 SsrA-tagged proteins is required for cell envelope stress resistance, then the major cellular
21 protease required for carrying out this activity (ClpP) would be necessary for survival. However,
22 in contrast to an *ssrA* deletion mutant (which is 100-fold or more sensitive to cell envelope
23 stress), $\Delta clpP$ cells exhibit only a modest cell envelope stress phenotype (3- to 5-fold more

1 sensitive, Fig. 5). This result suggests that the proteolysis of SsrA-tagged proteins by ClpXP is
2 at least partially dispensable with respect to cell envelope stress resistance, and implies that
3 ribosome release is the most critical aspect of the two SsrA functions.

4 To further examine this possibility, Δ ssrA cells were complemented with an allele (*ssrA^O*)
5 that is wild-type for ribosome release, but which contains a premature *ochre* stop codon that
6 gives rise to a truncated tag with reduced affinity to the proteolysis machinery (55). As
7 expected, the wild-type *ssrA* allele complements the SsrA phenotype (Fig. 5). Even though
8 SsrA^O is unable to target aborted polypeptides for proteolysis, the *ssrA^O* allele also largely
9 complements the SsrA phenotype, and cells containing SsrA^O are phenotypically similar to
10 Δ clpP cells (3- to 5-fold more sensitive, Fig. 5). These results suggest that while SsrA-mediated
11 proteolysis of aborted polypeptides is required to fully resist cell envelope stress, it is ribosome
12 release that is primarily responsible for allowing *E. coli* to survive under these environmental
13 conditions.

14 **Roles of MicA and RybB in conferring resistance to cell envelope stress.** The outer
15 membrane of a Gram-negative bacterium is studded with numerous β -barrel outer membrane
16 proteins that contribute to its structural integrity and govern its permeability (3). Two signal
17 transduction systems, σ^E and EnvZ-OmpR, employ sRNAs to downregulate OMP synthesis
18 during periods of stress. The σ^E pathway is activated by misfolded OMPs that accumulate in the
19 periplasm under conditions of cell envelope stress (52), and the EnvZ-OmpR system is
20 responsive to high osmolarity (reviewed in (37)). The sRNAs induced by σ^E and EnvZ-OmpR
21 halt OMP synthesis by blocking ribosome binding to OMP-encoding mRNAs and promoting the
22 degradation of the mRNAs (17). We were intrigued by the observation that a deletion mutant of
23 one σ^E -regulated sRNA, MicA, is severely sensitive to cell envelope stress while a deletion

1 mutant of another σ^E -regulated sRNA, RybB, is resistant. We thus examined whether mutants of
2 other OMP-regulating sRNAs exhibit cell envelope stress phenotypes that were missed in the
3 large-scale screen.

4 In agreement with the results of the large-scale experiments, *micC*, *micF*, and *cyaR*
5 deletion mutants did not show cell envelope stress phenotypes (Fig. 6). Individual *omrA* and
6 *omrB* deletion mutants also displayed wild-type phenotypes with respect to cell envelope stress
7 in the large-scale competition assay (Table S3). Since *omrA* and *omrB* are functionally
8 redundant and genetically linked (15, 16), we also tested cells that were doubly mutant for both
9 genes in addition to deletion mutants of two other OMP-regulating sRNAs, RseX and IpeX, that
10 were not initially included in our collection. None of these additional strains exhibited cell
11 envelope stress phenotypes (Fig. 6).

12 Finally, to test whether RybB and MicA act in the same pathway, we constructed a strain
13 that was doubly mutant for *micA* and *rybB*. If MicA and RybB were acting exclusively in the
14 same genetic pathways, then the double mutant would be expected to exhibit a C.I. value close to
15 the C.I. observed for one or the other single mutants. However, the double mutant exhibits an
16 intermediate C.I. of 0.11, compared to the *micA* (C.I. equal to 0.013) and *rybB* (C.I. equal to 2.2)
17 single mutants, and thus the two sRNAs most likely act independently of one another, possibly
18 through different sets of mRNA targets (Fig. 6).

19 **Acid stress screening experiments.** To examine the effects of another stress, the bar-
20 coded deletion collection was subjected to mock treatment and to acid shock. The data arising
21 from the acid shock experiments was analyzed as described above for the large-scale cell
22 envelope stress experiments. As with the cell envelope stress experiments, the rank order of the
23 most sensitive strains was roughly conserved in each of the three trials (Table S4). A mutant

1 deleted for a transcriptional activator of acid resistance genes in *E. coli*, *gadE* (28), appeared in
2 the top twenty most sensitive strains all six times. Seven additional deletion mutants (*yqgB*,
3 *mgrB*, *yobF*, *yceO*, *ylcG*, *hokE*, and *ybgT*) were among the most sensitive strains in at least four
4 of six possible instances. No deletion mutants appeared to be resistant to acid shock.

5 **Verification of acid shock sensitivity phenotypes.** We proceeded with one-on-one
6 competition assays after verifying that *lacZ* deletion mutants were wild-type with respect to acid
7 sensitivity (Fig. 7) and that *gadE* cells were acid-sensitive (C.I. equal to 0.0) (data not shown).
8 One-on-one competition experiments were performed with the *yqgB*, *mgrB*, *yobF*, *yceO*, *ylcG*,
9 and *hokE* mutant strains. As with the analyses of cell envelope stress phenotypes, we did not
10 further analyze the slow-growing *ybgT* deletion mutant. The results in Fig. 7 demonstrate that
11 *yqgB*, *mgrB*, *yobF*, and *yceO* deletion mutants are all severely sensitive to acid stress (mean C.I.
12 less than or equal to 0.2) while Δ *ylcG* and Δ *hokE* cells are not.

DISCUSSION

We have created a collection of 125 DNA bar-coded mutants in *E. coli*. 116 of these strains are deleted for genes encoding sRNAs and proteins of less than 70 amino acids, one strain is deleted for a known sRNA target, and the remaining eight strains are deleted for genes known to be necessary for resistance to various stress conditions. We were able to detect an array of phenotypes of varying severity, ranging from mutants that are very sensitive to cell envelope stress or acid shock to moderately sensitive and resistant cells. Even deletion mutations that give rise to moderate phenotypes are of considerable value, since they can be combined with one another to identify redundant genetic pathways.

Importantly, we were able to identify subtle deletion phenotypes which would remain undiscovered by more traditional methodologies. This is evidenced by the fact that none of the cell envelope stress sensitivity or resistance phenotypes are apparent when the corresponding mutant strains are incubated on LB agar plates containing 0.5% SDS and 1 mM EDTA (data not shown). This is in contrast to $\Delta smpA$ cells (the control strain known to be sensitive to cell envelope stress) which are readily distinguished from wild-type cells on such media (42).

Aside from the ability to detect subtle sensitivity and resistance phenotypes, the bar-coding approach we and one other group (39) have adapted to *E. coli* presents another advantage to traditional screening methodologies. Namely, the bar codes themselves, the microarray employed to detect them, and procedures to set up and analyze experiments have all been validated by the yeast community. Other groups have generated directed deletion mutants of almost every gene in *E. coli* (2), as well as some sRNA genes in *E. coli* (20) and *Salmonella* (33), but none of these collections incorporates DNA bar codes. In principle, MGK analysis could be employed to analyze our collection of bar-coded deletion mutants; however at present,

the chips employed in this methodology are not commercially available, have not tested been as extensively as the yeast bar code arrays used in our study (14, 35, 54), and would have to be custom-designed for our application (43).

Identification of novel cell envelope stress phenotypes. One-on-one competition experiments against otherwise wild-type *lacZ* mutant cells confirmed that eight deletion mutants arising from large-scale screens are indeed sensitive to cell envelope stress and two deletion mutants are resistant (Fig. 4). None of the cell envelope stress phenotypes we uncovered have been reported previously. Of particular note, we found that deletion mutants of two extensively studied genes, *ssrA* (10, 22) and *micA* (49), exhibit severe cell envelope stress phenotypes (Fig. 4A).

SsrA-mediated ribosome release is required for cell envelope stress resistance. SsrA acts in conjunction with the SmpB protein to mediate *trans*-translation, a process that frees stalled ribosomes (reviewed in (10)). In this process, SsrA exhibits two primary activities, protease tagging and ribosome release from mRNAs and aborted polypeptides. *ssrA* is essential in some bacteria (but not in *E. coli*) and is required for pathogenesis in *Yersinia* (10), survival of *Salmonella typhimurium* in macrophages (10), and swimming motility in *E. coli* (24). Deletion mutants of *ssrA* also induce an elevated heat shock response in *E. coli* (31).

We are the first to show a cell envelope stress sensitivity phenotype associated with the deletion of *ssrA*. Furthermore, two strains that are deficient in the proteolysis of SsrA-tagged proteins, $\Delta clpP$ and *ssrA*^O, show similar phenotypes and are only moderately sensitive to cell envelope stress (Fig. 5). This would imply that the more important activity of SsrA with respect to cell envelope stress resistance is ribosome release and not proteolysis tagging.

1 Many cell envelope proteins are co-translationally secreted at the inner membrane
2 (reviewed in (9)). When nascent polypeptides misfold during conditions of cell envelope stress,
3 it is conceivable that the activity of the secretory apparatus is inhibited, which would in turn halt
4 translation. Without SsrA, these membrane-bound ribosomes would remain stalled and
5 unavailable to participate in the response and possibly even contribute to cell envelope stress.

6 **MicA and RybB have opposite effects on cell envelope stress resistance.** $\Delta micA$ cells
7 are almost as sensitive to cell envelope stress as $\Delta ssrA$ cells (Fig. 4A). MicA is one of several *E.*
8 *coli* sRNAs that repress OMP translation (49). The MicA phenotype we observed is striking
9 given that deletion mutants of seven other genes coding for OMP-regulating sRNAs (*omrAB*,
10 *micC*, *micF*, *ipeX*, *cyaR*, and *rseX*) did not exhibit cell envelope stress phenotypes in our study
11 (Fig. 6, Table S3) and the deletion mutant of one OMP-regulating sRNA (*rybB*) exhibited
12 resistance (Fig. 6).

13 It is curious that $\Delta micA$ cells are extremely sensitive to cell envelope stress, while $\Delta rybB$
14 cells are resistant. One might expect that $\Delta rybB$ cells are more resistant because they upregulate
15 σ^E activity (47); however, this also occurs in *micA* mutants (Fig. S1). Given that MicA and
16 RybB target different sets of mRNAs in *Salmonella* (34) one plausible explanation for the RybB
17 resistance phenotype is that the synthesis of an OMP or OMPs that make(s) the cell more
18 resistant to cell envelope stress is upregulated in $\Delta rybB$ but not in $\Delta micA$ cells due to de-
19 repression. Alternatively, RybB might normally upregulate genes that are detrimental to
20 surviving cell envelope stress.

21 **Identification of novel acid shock phenotypes.** Deletion mutants of four genes coding
22 for small proteins (*yobF*, *yceO*, *mgrB*, and *yqgB*) were confirmed to exhibit novel acid sensitivity
23 phenotypes (Fig. 7). *mgrB* exhibits regulation that is consistent with its acid-sensitive deletion

1 phenotype. This gene was so named because it is regulated by the PhoQ-PhoP two-component
2 system (21), which is responsive to low concentrations of Mg^{2+} (30). Expression of *mgrB* is also
3 activated by the EvgS-EvgA two-component system in a PhoP-dependent manner (11).
4 Although it is unclear what stimulates the sensor kinase EvgS *in vivo*, artificial activation of
5 *evgA* has been reported to make exponentially growing *E. coli* cells acid-resistant (29). The
6 YqgB sensitivity phenotype could arise as a consequence of polarity effects on two downstream
7 genes, *speA* and *speB*. SpeA and SpeB are required for the synthesis of polyamines (4), the
8 presence of which has been shown to confer acid resistance to *E. coli* (41, 56). The *yqgB* gene
9 contains an internal promoter that drives expression of *speA* and *speB* (44). Therefore, deleting
10 *yqgB* could eliminate *speAB* expression and render the *yqgB* mutant cells acid-sensitive. Another
11 group has recently reported that $\Delta gcvB$ cells are acid-sensitive (20). We did not observe this
12 phenotype (Table S4); however, we exposed cells to pH 1.8 for 10 min, while Jin *et al.* exposed
13 cells to pH 2.0 for 30 min.

14 **Overlap of phenotypic data with regulation of small proteins.** A large number of
15 small protein-encoding genes are regulated by changes in growth or stress conditions (18).
16 Deletion mutants of this set of stress-regulated small protein-encoding genes did not exhibit cell
17 envelope or acid shock phenotypes in the present study. This is perhaps not surprising since the
18 acid shock and cell envelope stress conditions employed in the two studies were slightly
19 different. Additionally, two genes (*yohP* and *yshB*) that were shown to be induced in response to
20 cell envelope stress, were not included in our collection of bar-coded deletion mutants (18).
21 However, a deletion mutant of *yobF* (which is post-transcriptionally induced by heat shock (18))
22 is moderately sensitive to cell envelope stress and severely sensitive to acute acid stress (Fig. 4A
23 and Fig. 7). Absent any polarity effects on the downstream gene encoding a cold-shock protein,

1 *cspC*, these data suggest that YobF warrants further investigation as a potential component of a
2 generalized stress-response pathway.

3 **Future directions.** Until recently, the large number of sRNAs and small proteins
4 encoded in the intergenic regions of bacterial genomes has been underappreciated. Advances in
5 bioinformatic approaches, the development of densely tiled oligonucleotide microarrays, and
6 cloning-based approaches coupled with DNA pyrosequencing technology are extending the list
7 of sRNA and small protein genes of undefined function. Deletion mutants of these newly
8 discovered genes can be readily added to our bar-coded collection and tested *en masse* under
9 conditions of cell envelope stress, acid shock, and most any other stress condition (e.g. alkaline
10 stress, ethanol stress, heavy metal stress) for novel phenotypes. The phenotypes uncovered in
11 these assays will facilitate genetic studies as well as the application of biochemical and
12 cytological methodologies to further illuminate the roles sRNAs and small proteins play in the
13 cell.

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FIGURE LEGENDS

FIG. 1. Diagram of bar-coded antibiotic resistance cassettes. Kanamycin resistance cassettes flanked by two unique 20-mer DNA bar code sequences (“UP” and “DN”) were generated by a two-step PCR process for each deleted gene. The bar-coded kanamycin resistance cassettes were incorporated at loci coding for sRNAs and small proteins by homologous recombination. For the analysis of the large-scale competition experiments, bar codes upstream and downstream of every kanamycin resistance cassette were amplified by means of common primer sequences (indicated by small black arrows) encoded within the regions bordering the UP and DN bar codes. The amplified bar codes were then hybridized to a DNA microarray to score each bar-coded deletion mutant within the population.

FIG. 2. Most strains have no membrane stress phenotype under conditions of cell envelope stress. A representative histogram of relative abundance (R.A.) values obtained from measurements of the fluorescence intensities of the “DN” tags in one experiment shows that the majority of strains have an R.A. value close to one (denoted by a solid black line). This indicates that they exhibit no phenotype under conditions of cell envelope stress. Sensitive strains have the lowest R.A. values, while resistant strains have the highest R.A. values.

FIG. 3. Small-scale competition assays illustrate a range of phenotypes. Otherwise wild-type LacZ⁻ cells (NM601) were competed against one of four LacZ⁺ competitor strains: *Δblr* (GSO280), wild-type *E. coli* K-12 MG1655, *ΔsroH* (GSO278), or *ΔssrA* (GSO279) as described in the Materials and Methods. The total numbers of blue and white colonies vary in the mock treated samples (Mock) but the ratio of blue to white colonies is roughly 1:1 in all instances. For

1 wild-type cells, this ratio is unchanged in the stress treated sample (Stress). However, *blr*
2 mutants are more resistant to cell envelope stress than *lacZ* mutants, as evidenced by the
3 preponderance of blue colonies in the corresponding stress-treated sample. In contrast, *sroH* and
4 *ssrA* mutant cells are sensitive to cell envelope stress as shown by the lack of blue colonies
5 relative to white colonies. The calculated competitive indices (C.I.) for these individual
6 experiments are provided beneath each strain name.

7
8 FIG. 4. sRNA and small protein deletion mutants sensitive or resistant to cell envelope stress. In
9 panels A and B, competitor strains were grown in competition with *LacZ*⁻ cells (NM601) under
10 mock treatment conditions or conditions of cell envelope stress as described in the Materials and
11 Methods. A competitive index (C.I.) was calculated for each experiment; the C.I. values
12 reported for all strains are the means of three trials, except for MG1655 (n = 4). The error bars
13 represent one standard deviation from the mean. Wild-type MG1655 cells did not exhibit a cell
14 envelope stress phenotype and were employed as controls in both panels. (A) Cells mutant for
15 *ssrA* (GSO279), *ybaM* (GSO283), *micA* (GSO271), *ryeF* (GSO277), *yqcG* (GSO288), *sroH*
16 (GSO278), *ybhT* (GSO284), or *yobF* (GSO287) were sensitive to cell envelope stress. Cells
17 mutant for *glmY* (GSO269) exhibited very modest sensitivity to cell envelope stress, while *yqgB*
18 (GSO289) deletion mutants were effectively wild-type. (B) Cells mutant for *blr* (GSO280) or
19 *rybB* (GSO276) are resistant to cell envelope stress. (C) Complemented *LacZ*⁻ deletion mutants
20 of *ssrA* (GSO298), *ybaM* (GSO299), *micA* (GSO297), and *ybhT* (GSO300) and uncomplemented
21 *LacZ*⁻ deletion mutants of *ssrA* (GSO294), *ybaM* (GSO295), *micA* (GSO293), and *ybhT*
22 (GSO296) were competed against *LacZ*⁺ wild-type MG1655 cells as described in the Materials
23 and Methods.

1 FIG. 5. Strains deficient in SsrA-mediated proteolysis are only moderately sensitive to cell
 2 envelope stress. Uncomplemented LacZ⁻ deletion mutants of *ssrA* (GSO294) (n=4) as well as
 3 deletion mutants of *ssrA*⁺ complemented with either a wild-type allele of *ssrA* [*ssrA*⁺ (GSO301)]
 4 (n=6) or an *ochre* codon mutant [*ssrA*^O (GSO302)] (n=5) were competed against LacZ⁺ wild-
 5 type MG1655 cells. A deletion mutant of *clpP* (GSO303) was also competed against LacZ⁻
 6 (NM601) cells (n=3). A competitive index (C.I.) was calculated for each experiment. The error
 7 bars represent one standard deviation from the mean.

8
 9 FIG. 6. Deletion mutants of most OMP-regulating sRNAs exhibit wild-type cell envelope stress
 10 phenotypes. Otherwise wild-type LacZ⁻ mutant cells (NM601) were competed against one of
 11 nine LacZ⁺ competitor strains: $\Delta micA$ (GSO271), $\Delta micA \Delta rybB$ (GSO290), $\Delta omrAB$ (GSO274),
 12 $\Delta micC$ (GSO272), $\Delta micF$ (GSO273), $\Delta rseX$ (GSO275), $\Delta ipeX$ (GSO270), $\Delta cyaR$ (GSO268), and
 13 $\Delta rybB$ (GSO276) as described in the Materials and Methods. Wild-type MG1655 cells did not
 14 exhibit a cell envelope stress phenotype and were employed as a control. A competitive index
 15 (C.I.) was calculated for each experiment; the C.I. values reported for each strain are the means
 16 of three trials, except for MG1655 (n = 4). The error bars represent one standard deviation from
 17 the mean.

18
 19 FIG. 7. Four small protein deletion mutants are sensitive to acid stress. Otherwise wild-type
 20 LacZ⁻ mutant cells (NM601) were competed against one of seven LacZ⁺ competitor strains:
 21 $\Delta yqgB$ (GSO289), $\Delta mgrB$ (GSO282), $\Delta yobF$ (GSO287), $\Delta yceO$ (GSO285), $\Delta ylcG$ (GSO286),
 22 $\Delta hokE$ (GSO281) and wild-type MG1655 as described in the Materials and Methods. A
 23 competitive index (C.I.) was calculated for each experiment; the C.I. values reported for each

- 1 strain are the means of three trials, except and $\Delta ylcG$ (GSO286) and $\Delta hokE$ (GSO281) ($n = 5$).
- 2 The error bars represent one standard deviation from the mean.













